Cell, Vol. 17, 683–689, July 1979,

Isolation of Adenovirus Type 5 Host Range Deletion Mutants Defective for Transformation of Rat Embryo Cells

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Summary

A series of adenovirus type 5 (Ad5) deletion, insertion and substitution mutants, some of which are defective for transformation of rat cells, have been isolated. The mutants were selected as variants which lack the Xba I endonuclease cleavage site at 4 map units on the viral chromosome. The deletions range in size from 150–2300 bp and are located between 1.5 and 10.5 map units. The mutants can be propagated in 293 cells (Ad5-transformed human embryonic kidney cells), but are defective for growth in HeLa or human embryonic kidney cells. No viral DNA synthesis was observed in mutant virus-infected HeLa cells. All but one of the deletion mutants tested were defective for transformation of rat embryo and rat embryo brain cells.

Introduction

Several lines of evidence indicate that the adenovirus gene(s) which mediate cellular transformation are located at the left end of the conventional adenovirus genetic map. Ad2 left end sequences are consistently present (Gallimore, Sharp and Sambrook, 1974) and transcribed (Flint, Gallimore and Sharp, 1975; Flint et al., 1976) in transformed rat cells. Other regions of the viral genome need not be present. Furthermore, rat kidney cells can be transformed using a 0.08 fractional length DNA fragment (Hind III fragment G) derived from the left molecular end of the viral chromosome (Graham et al., 1974).

Mutations in the left end region of the viral genome should help to delineate the role of viral gene functions in cellular transformation. Very few mutants have been isolated which contain lesions in this region. Many temperature-sensitive mutants of Ad2 and Ad5 have been isolated (see Ginsberg and Young, 1977), and several of these mutations affect the frequency of viral transformation (Wilkie, Ustacelebi and Williams, 1973; Ginsberg et al., 1974), but none map in the left end 8% of the viral genome. Several host range mutants have been isolated whose lesions are located in this region (Harrison, Graham and Williams, 1977). These mutants probably contain single base pair substitutions and form two complementation groups. One group maps between 0 and 4.5 map units by marker rescue; the other maps between 0 and 24 map units (Frost and Williams, 1978).

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We have recently described a protocol for the isolation of mutants which preexist in viral DNA preparations (Jones and Shenk, 1978). This procedure is based on the loss of restriction endonuclease cleavage sites. We have used this procedure to isolate Ad5 mutants which lack the Xba I endonuclease cleavage site at 4 map units. These mutants enjoy the advantages of both conditional-lethal and deletion mutations. They grow to wild-type level titers on 293 cells (Ad5-transformed human embryonic kidney cells; Graham et al., 1977) but will not replicate in HeLa cells; they contain substantial alterations which are easily mapped; and they are nonleaky and thus can be used to infect HeLa cells at high multiplicity to investigate the molecular nature of their defects.

Results

Isolation of Xba I Endonuclease-Resistant Mutants

Xba I cleaves Ad5 DNA at four sites: 4, 29, 79 and 85 map units (Figure 1a). The site at 4 map units is located within the left end region of the Ad5 genome which contains a gene (or genes) responsible for cellular transformation. Our objective was to isolate mutants lacking this cleavage site.

Mutants lacking restriction endonuclease cleavage sites can be isolated from a population of wild-type Ad5 DNA (Jones and Shenk, 1978). To select mutants, a preparation of Ad5 DNA is cleaved with a restriction endonuclease and the fragments are rejoined with DNA ligase. The modified DNA is used to infect cells in a DNA plaque assay and the resulting virus clones are screened for mutants which lack the cleavage site recognized by the restriction endonuclease. This procedure exerts a strong selection for such molecules because fewer ligation events are required to regenerate a complete mutant DNA molecule (it was initially cut into fewer pieces) than to produce an intact wild-type DNA.

This selection procedure is useful only for restriction endonucleases which cleave Ad5 DNA at a limited number of sites. If too many fragments are generated, infectious DNA molecules will not be reassembled at a detectable level. For this reason we used *sub*304 DNA as our starting material to select Xba I-resistant variants. This viable substitution mutant was selected as lacking the Eco RI cleavage site at 83.5 map units (Jones and Shenk, 1978). Its deletion extends from 83 to 85 map units, and it lacks the Xba I cleavage site at 85 map units. As a result, *sub*304 DNA is cleaved at only three sites by Xba I, generating a fused D/C fragment (Figure 1b).

*Sub*304 DNA was cleaved with Xba I, the fragments were rejoined with T4 DNA ligase and the modified DNA was used to infect cells in a DNA plaque assay. Only one mutant (308) was isolated in this experiment. The DNA of this mutant is cleaved at only two sites by

EXHIBIT C

Xba I, generating B, E and a fused A/D/C fragment (Figure 1b). Thus mutant 308 lacks both the 85 and 79 map unit cleavage sites. It is viable, and the alteration at 79 map units is either a very small deletion or a single base pair substitution.

Another round of mutant selection was carried out by cleaving and ligating mutant 308 DNA. The two mutants selected in this experiment were 309 and 311. When mutant 309 DNA was cleaved with Xba I, two fragments were generated: an E fragment and fused B/A/D/C fragment (Figure 1b). Thus mutant 309 DNA lacks the cleavage site at 29 map units in addition to the sites at 79 and 85 map units. Again, the mutant is viable and its alteration at 29 map units is either a small deletion or single base pair substitution. Mutant 311 was the first of the class of mutants for which we were searching. This DNA was cleaved by Xba I to generate two fused fragments: an E/B and an A/D/C fragment (Figure 1b). Thus this mutant DNA lacks the cleavage site at 4 map units in addition to the sites at 79 and 85 map units.

Mutant 309 DNA was then cleaved with Xba I and the cleaved DNA was used to infect cells in a DNA plaque assay. Only Xba I-resistant DNAs remained intact and infectious after cleavage. Since mutant 309 DNA contains only the 4 map unit cleavage site, this manipulation enabled us to isolate easily a series of mutants lacking that site.

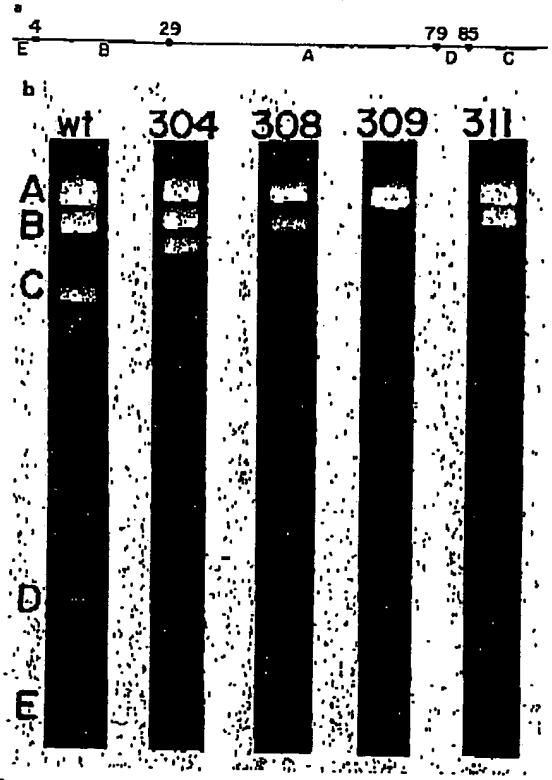

Figure 1. Electrophoretic Analysis of Fragments Produced by Cleavage of Wild-Type and Mutant DNAs with Xba I

(a) Xba I cleavage map of Ad5 DNA. (b) DNAs (0.5 μg) were cleaved and the resulting fragments were analyzed by electrophoresis for 16 hr at 40 V on 0.8% agarose gels.

Deletion, Insertion and Substitution Mutants Were Isolated

The mutants which lack the Xba I cleavage site were first analyzed by cleavage with Hind III (Figure 2). Mutants lacking the Xba I site at 4 map units should contain alterations within the Hind III-G fragment (the segment from 0 to 8 map units). The Hind III-G fragments of mutants 311 and 312 migrate faster than the corresponding wild-type fragment (Figure 2), indicating that they are smaller than the wild-type fragment. Digestion of mutant 313 and 315 DNAs with Hind III produced fused E/G fragments, indicating that these mutants contain deletions which extend from the Xba I site at 4 map units across the Hind III site at 8 map units (the junction between the Hind III-G and -E fragments). When heteroduplex DNA molecules were prepared between mutant 315 and wild-type DNAs and examined in the electron microscope (data not shown), they were found to contain substitution loops. Thus mutant 315 lacks a segment of adenovirus DNA which includes the Xba I and Hind III cleavage sites at 4 and 8 map units, respectively, and has acquired a new DNA segment in this region.

The molecular structures of the mutant genomes are diagramed in Figure 3. These structures were

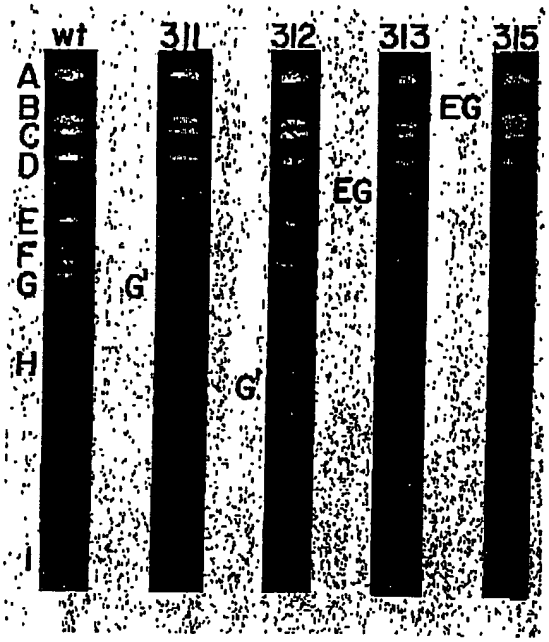

Figure 2. Electrophoretic Analysis of Fragments Produced by Cleavage of Wild-Type and Mutant DNAs with Hind III

DNAs (0.5 μg) were cleaved and the resulting fragments were analyzed by electrophoresis for 16 hr at 40 V on 0.8% agarose gels.

deduced from restriction endonuclease cleavage analysis and electron microscopic heteroduplex mapping. Mutant 310 lacks the Xba I site at 4 map units, but we did not detect a deletion in this mutant when a fragment as small as Hpa I-E (0–4.5 map units) was examined. A small deletion could have gone undetected; alternatively, these mutants may simply contain a single base pair change within the recognition site of the endonuclease. Mutant *dl*311 lacks about 150 bp; this deletion occurs between the Sma I and Hpa I cleavage sites (3.5 and 4.5 map units, respectively) which are both present in *dl*311 DNA. Mutant *dl*312 lacks 1030 bp. The right end boundary of this deletion lies between the Xba I site at 4 map units, which is missing, and the Hpa I site at 4.5 map units, which is present; the left end is placed at 1.5 map units both by subtracting 2.9 map units from the right end coordinate (1 map unit is 360 bp) and by measuring the position of the deletion loop in *dl*312 × *wt*300 heteroduplex DNAs. Mutant *dl*313 lacks 2350 bp. The left end boundary of this deletion lies between the missing Xba I site at 4 map units and the Sma I site at 3.5 map units (which is present). Measurement of the position of the deletion loop in *dl*313 × *wt*300 heteroduplex DNAs places the right end boundary at 10.5 map units; the Sma I site at 11 map units is present. Mutant *dl*314 lacks 430 bp; the Sma I site at 3.5 map units is present, and both the Xba I and Hpa I sites (at 4 and 4.5 map units, respectively) are missing. Two substitution mutants were isolated. *Sub*315 lacks 1780 bp. The Sma I site at 3.5 map units is present; all cleavage sites from the Xba I site at 4 map units through the Hind III site at 8 map units are missing. *Sub*316 lacks 820 bp; this deletion occurs between the Sma I and Kpn I cleavage sites (3.5 and 6.0 map units), which are both present. Both substitution mutants contain DNA segments that appear to have been inserted at the location of the deletions. The origin of these inserted segments is unknown. Finally, *in*317 contains a 360 bp insertion with no evidence of a deletion. Again, the origin of the inserted segment is unknown. This mutant does not lack the Xba I site at 4 map units, and was apparently isolated by chance.

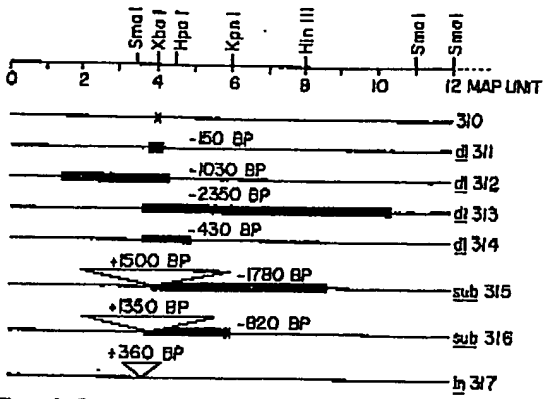

Figure 3. Portion of the Ad5 Physical Map (0–12 Map Units) Showing the Alterations Present in the Mutant DNAs

The nature of each alteration was determined from the data in Figure 2, digestion with the restriction endonucleases indicated at the top of the physical map (discussed in Results) and by electron microscopic heteroduplex mapping (discussed in Results). Ad5 segments which have been deleted are indicated by heavy bars; insertions are designated by triangles. Although the deletions in *dl*312 and *dl*313 (and others) are drawn as if they overlap, it is conceivable that they do not. Both mutants lack the Xba I cleavage site at 4 map units. It is improbable, but not impossible, that they lack nonoverlapping portions of the 6 bp recognition site of the enzyme. It is not known whether the mutation in mutant 310 is a very small deletion or a single base pair change. This mutation is marked with an "x".

Deletion and Substitution Mutants Are Defective for Growth in HeLa and Human Embryonic Kidney Cells

The mutants described here were all isolated and propagated in 293 cells (Ad5-transformed human embryonic kidney cells; Graham et al., 1977). These cells contain and express the left end 11% of the Ad5 genome (Graham et al., 1977); as a result they are able to complement Ad5 mutants which are defective due to alterations within this left end region.

To compare the growth of the mutants in Ad5-transformed 293 cells to non-Ad5-transformed human cells (HeLa cells), stocks of the viruses were diluted and plated on both cell types in a plaque assay (Table 1). The *wt*300 and mutant 310 viruses did not exhibit host range phenotypes; they produced a roughly equivalent number of plaques on both cell types. The *dl*311 virus stock produced about the same number of plaques as the wild-type on 293 cells, but the plaque number was reduced about 1000 fold on HeLa cells. While all the mutants with larger deletions than that in *dl*311 produced wild-type levels of plaques on 293 cells, they were all markedly defective for growth in HeLa cells. Mutants *dl*312, *dl*313 and *sub*315 produced no detectable plaques on HeLa cells. At a multiplicity between 1 and 10 pfu per cell, these mutants killed the HeLa cell monolayers but no individual plaques were observed. Mutants *dl*314 and *sub*316 produced a few plaques on HeLa cells, but only about 10^{-7} the number produced on 293 cells. Virus from plaques generated on HeLa cells by these mutants (or by *dl*311) exhibited the same host range phenotype as the original stock from which they were derived. Hence these plaques are not due to the presence of revertants in the virus stocks.

The growth of the insertion mutant, *in*317, was only moderately restricted in HeLa cells (Table 1). Its titer differed only about 100 fold between permissive and nonpermissive cells.

Several of the mutant stocks were also tested for plaque production on primary human embryonic kidney (HEK) cells (Table 1). The *dl*313 and *sub*315 stocks produced a small number of plaques on these cells. Evidently a cellular function expressed in HEK

Table 1. Host Dependence of Mutants

Virus	Deletion Size, Location[a]	Titer (pfu) 293 Cells	HeLa Cells	HEK Cells
*wt*300	—	1.3×10^9	1.2×10^9	4.2×10^9
310	—	3.0×10^9	1.7×10^9	1.6×10^9
*dl*311	-150 bp, 3.5–4.5	5.0×10^9	7.0×10^{8b}	1.5×10^9
*dl*312	-1030 bp, 1.5–4.5	3.0×10^9	$<5 \times 10^2$	$<10^2$
*dl*313	-2350 bp, 3.5–10.5	1.2×10^9	$<5 \times 10^2$	2.5×10^5
*dl*314	-430 bp, 3.5–5.0	2.7×10^9	5.0×10^{3b}	NT[c]
*sub*315	-1780 bp, 3.5–8.5	4.5×10^9	$<5 \times 10^2$	6.0×10^6
*sub*316	-280 bp, 3.5–6.0	3.1×10^9	5.0×10^3	NT[c]
*in*317	—	4.5×10^9	2.5×10^{7b}	NT[c]

[a] Location indicates the boundaries of the deletion (±0.5 map unit) on the conventional Ad5 map.
[b] Several HeLa cell plaque isolates were grown into stocks on 293 cells and found to have the same host range phenotype as their parent.
[c] NT = not tested.

but not in HeLa cells is partially able to complement the defects in these mutants. Similar observations have been made with host range mutants of polyoma (Benjamin and Goldman, 1974) and adenovirus (Harrison et al., 1977). The *dl*312 virus stock produced no plaques on HEK cells. It is probable that the different response on HEK cells exhibited by *dl*312 as compared to *dl*313 and *sub*315 is due to the fact that *dl*312 (lacks a DNA segment mainly to the left of 4 map units; Figure 3) and *dl*313 and *sub*315 (lack segments mainly to the right of 4 map units; Figure 3) affect different viral functions. This notion is strengthened by the fact that *dl*312 and *dl*313 complement each other in mixed infections of HeLa cells (see below and Figure 5).

Host Range Deletion Mutants Do Not Synthesize Viral DNA in Infected HeLa Cells

To evaluate the ability of the host range mutants to synthesize viral DNA in nonpermissive cells, mutant-infected cells were labeled 6–30 hr after infection. Total cellular DNA was prepared and a portion was centrifuged to equilibrium in CsCl. Whereas *dl*312- or *dl*313-infected 293 cells contained a prominent ^{3}H-labeled species with the density characteristic of Ad5 DNA (1.715 g/cc) in addition to ^{3}H-labeled cellular DNA (1.705 g/cc), no viral DNA was evident in infected HeLa cells (Figure 4). We conclude that the blocks in the mutant growth cycle prevent normal viral DNA synthesis.

Transformation by Deletion Mutants

The ability of mutant virus to induce focus formation in infected rat cells was investigated. Cells were infected, passaged the following day at a 1:6 dilution, allowed to grow in normal medium until complete monolayers formed and then maintained in a low Ca^{++} medium. In this medium, transformed cells continue to divide and form foci; nontransformed cells do not grow. Primary rat embryo cells were infected in the first assay (Table 2). No foci were evident on plates containing uninfected cells, although cultures infected with *wt*300, *sub*304 (the parent of all the host range mutants, lacking the region from 83–85 map units), 310 and *dl*311 all contained foci of transformed cells. In contrast, no foci were identified on monolayers infected with *dl*312, *dl*313 or *sub*315. Similar results were obtained in a second assay performed with embryonic rat brain cells (Table 2). Using focus formation as our criterion, we conclude that *dl*312, *dl*313 and *sub*315 are defective for transformation.

Several of the *wt*300- and *dl*311-transformed rat embryo cell foci have been propagated through ten subclonings. The morphological characteristics and growth rates of the two types of transformants are indistinguishable. The 150 bp deletion in *dl*311 occurs within a region represented in early cytoplasmic mRNAs (Jones and Shenk, 1979). Apparently this region is not essential for transformation.

Mutants *dl*312 and *dl*313 Complement for Lytic Growth

When HeLa cells are infected with either *dl*312 or *dl*313 virions alone, no virus yield is produced (Figure 5). Co-infection of HeLa cells with these two mutants, however, generates a mixed yield of the two input mutants (Figure 5). We conclude that *dl*312 and *dl*313 complement for lytic growth and thus lack different functions.

Discussion

The deletion mutants described in this paper display host range phenotypes. They do not replicate in HeLa cells, but produce wild-type yields in 293 cells (Table 1). Harrison et al. (1977) have also reported the

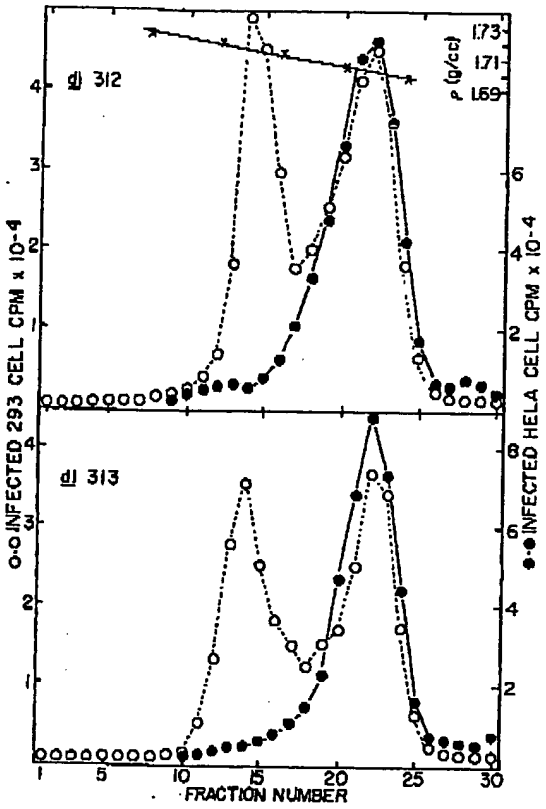

Figure 4. Analysis by Equilibrium Density Centrifugation of DNAs Synthesized in *dl*312- and *dl*313-Infected Cells

HeLa or 293 cells (one 100 mm diameter plate containing about 10^7 cells) were infected with each mutant virus at a multiplicity of 40 pfu per cell and labeled with ^{3}H-thymidine (50 μCi per plate) 6–42 hr after infection. DNA was prepared by digestion of nuclei with proteinase K [50 μg/ml in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% SDS, 37°C, 5 hr] followed by phenol extraction. ^{32}P-labeled Ad5 DNA (1000 cpm) was added to a portion of each cellular DNA preparation and the samples were centrifuged to equilibrium in CsCl [1.70 g/cc in 10 mM Tris–HCl (pH 8), 1 mM EDTA]. Aliquots were collected and their radioactivity counted. Data from two gradients [(O—O) infected 293 cells, (●—●) infected HeLa cells] are shown in each panel. They were superimposed by aligning the peaks of ^{32}P-labeled Ad5 DNA. The Ad5 marker DNAs (not shown in figure) banded at precisely the location of the 1.717 g/cc peaks derived from virus-infected 293 cells. Uninfected 293 cells did not contain detectable amounts of DNA which banded at this position.

isolation of Ad5 host range mutants. Their mutants were generated by nitrous acid and ultraviolet light mutagenesis, and they were screened from the mutagenized population for their ability to grow on 293 but not on HeLa cells. Their host range mutants formed two complementation groups. A representative group I mutant (*hr*1) has been mapped by marker rescue experiments. It lies within the Hpa I-E fragment (0–4.5 map units on the Ad5 chromosome; Frost and Williams, 1978). Thus far the phenotype of *hr*1 appears similar to that of *dl*312, whose deletion is between 1.5 and 4.5 map units (Figure 3). Both mutants are defective for growth in HeLa cells; both fail to

Table 2. Transformation Assays on Rat Embryo and Rat Brain Cells

Virus	Deletion Size, Location[a]	Foci on Rat Embryo Cells[b]	Foci on Rat Brain Cells[c]
Uninfected	—	0	0
*wt*300	—	48	36
*sub*304	—	66	NT[d]
310	—	83	16
*dl*311	-150 bp, 3.5–4.5	22	32
*dl*312	-1030 bp, 1.5–4.5	0	0
*dl*313	-2350 bp, 3.5–10.5	0	0
*sub*315	-1780 bp, 3.5–8.5	0	0

[a] Location indicates the boundaries of the deletion on the conventional Ad5 map.
[b] The total number of foci on twelve 60 mm diameter plates of primary rat embryo cells are tabulated.
[c] The total number of foci on six 60 mm diameter plates of primary rat brain cells are tabulated.
[d] NT = not tested.

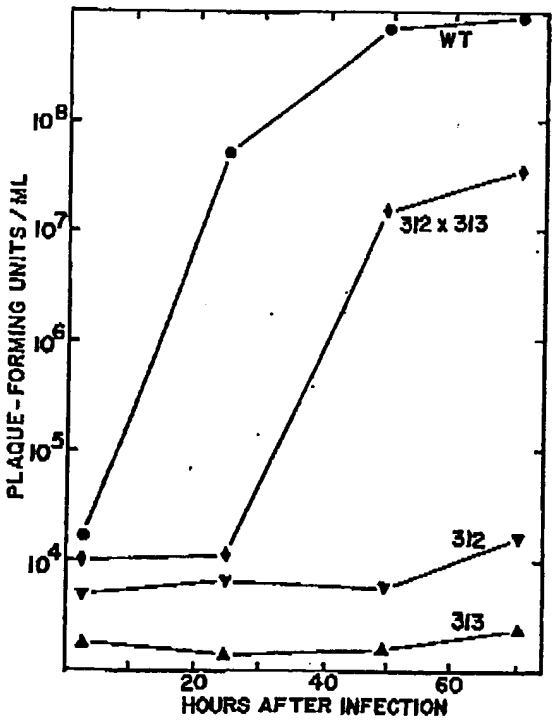

Figure 5. Complementation Analysis of *dl*312 and *dl*313 Viruses in HeLa Cells

HeLa cells were infected at a multiplicity of 20 pfu per cell with either virus alone, or co-infected at a multiplicity of 10 pfu per cell with each of the two viruses. After adsorption for 60 min at 37°C, the monolayers were washed twice with Tris-buffered saline, and medium containing 5% fetal calf serum was added. Cultures were harvested at the indicated times and the virus titer was measured by plaque assay on 293 cells. The absence of wild-type recombinants in the 60 hr *dl*312 × *dl*313 yield was established by plaque assay on HeLa cells. (▼) *dl*312 alone; (▲) *dl*313 alone; (◆) *dl*312 × *dl*313; (●) *wt*300 alone.

synthesize viral DNA in HeLa cells (*hr*1, Harrison et al., 1977; also *hr*3, Lassam, Bayley and Graham, 1978; *dl*312, Figure 4); and both fail to transform whole rat embryo or rat embryo brain cells at wild-type frequencies (*hr*1, Graham, Harrison and Williams, 1978; *dl*312, Table 2). The principal difference between *hr*1 and *dl*312 is the magnitude of their host range effects. The ratio of plaque formation on HeLa as compared to 293 cells is about 3×10^{-4} for *hr*1 (Harrison et al., 1977) and $<10^{-7}$ for *dl*312 (Table 1). Assuming that a single base pair change is responsible for the *hr*1 phenotype, it would probably be more "leaky" than a deletion mutant. The exceptionally "tight" phenotype displayed by *dl*312 (and several of the other deletion mutants), however, is probably due in part to inactivation of multiple viral functions. Mutant *dl*312 lacks a DNA segment which appears to code for portions of several functions (multiple, overlapping Ad2 transcripts have been mapped within this region; Chow et al., 1977; Berk and Sharp, 1978).

The relationship between the group II host range mutants (such as *hr*6) of Harrison et al. (1977) and the mutants reported here is less clear. Mutant *hr*6 has been mapped by marker rescue within the Sal I-B fragment (0–24.5 map units; Frost and Williams, 1978). This mutant is defective for growth in HeLa cells, but grows as efficiently in untransformed HEK cells as in 293 cells (Harrison et al., 1977). In this respect *hr*6 resembles *dl*313 and *sub*315, whose defects are partially complemented in HEK cells (Table 1); *hr*6, however, synthesizes viral DNA in the nonpermissive HeLa cells (Lassam et al., 1978) while *dl*313 and *sub*315 fail to do so (data for dl313 in Figure 4). Complementation and recombinational analyses should help to clarify the relationship, if any, of *dl*313 and *sub*315 to the group II mutants of Harrison et al. (1977).

As shown in Figure 5, *dl*312 and *dl*313 complement for lytic growth in HeLa cells. This result was not anticipated because both mutants lack the Xba I site at 4 map units, and it is probable that the deletions overlap. Nevertheless, the mutations complement, and it follows that they alter different lytic functions of the virus. Since both mutants are unable to synthesize DNA in infected HeLa cells, we conclude that there are at least two gene products located between 1.5 and 10.5 map units which are required for viral DNA synthesis. Thus at least four gene products are required for Ad5 DNA synthesis: the *hr*1/*dl*312 product (1.5–4.5 map units; Harrison et al., 1977; Frost and Williams, 1978; Figure 3), the *dl*313 product (3.5–10.5 map units; Figure 3), the *ts*36/*ts*149 gene product (9.4–24 map units; Williams, Young and Austin, 1974; Ginsberg et al., 1974; Frost and Williams, 1978; *dl*313 and *ts*36 complement, W. Colby and T. Shenk, unpublished results) and the *ts*125 product (60–64 map units; Ginsberg et al., 1974; Frost and Williams, 1978). Finally, since *dl*312 and *dl*313 complement for lytic growth and are both defective for transformation, it is possible that multiple genes are required for transformation. Experiments designed to test this notion by asking whether these two mutants complement in transformation assays are in progress.

Analysis of the molecular defects of the host range deletion mutants has produced several interesting results. Mutant *dl*312-infected HeLa cells contain no detectable early cytoplasmic mRNAs (Jones and Shenk, 1979). There are at least four regions of early transcription, and each region contains at least one promoter (Evans et al., 1977, Berk and Sharp, 1978). Thus the *dl*312 mutation evidently defines an early viral gene product required for expression of other early gene products encoded by distant regions of the chromosome.

Mutant *dl*313 lacks a portion of the gene coding for protein IX, a hexon-associated virion polypeptide (Everitt et al., 1973) whose mRNA has been located between 9.5 and 11.2 map units on the viral chromosome (Pettersson and Mathews, 1977). Mutant *dl*313 virions produced in 293 cells contain no protein IX (W. Colby, N. Jones and T. Shenk, unpublished data). Protein IX is apparently a nonessential component of the virion. Mutant *dl*313 virions, however, are more thermolabile than wild-type virions.

Experimental Procedures

Cells, Virus and Plaque Assays

The 293 cell line (an Ad5-transformed human embryo cell line) was provided by F. Graham and has been described (Graham et al., 1977). The cells were maintained in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal calf serum. HeLa cells were obtained from J. Williams. They were grown in DMEM containing 5% fetal calf serum. Human embryonic kidney cells were purchased from Microbiological Associates. These cells were propagated in DMEM containing 10% fetal calf serum. The cells obtained from the vendor were passaged (1:5) twice before use in plaque assays. Whole rat embryo and rat embryo brain cells were prepared from 21 day embryos by dissection and trypsinization. The cells were propagated in DMEM containing 10% fetal calf serum and used for transformation assays without further subculturing. The wild-type Ad5 (H5 *wt*300) was a plaque-purified derivative of a virus stock originally obtained from H. Ginsberg. Mutant H5 *sub*304 was selected for loss of the Eco RI cleavage site at 83.5 map units (Jones and Shenk, 1978). This mutant lacks the region of the Ad5 chromosome between 83 and 85 map units and contains an insertion of cellular DNA at this site. The $(Ca)_3(PO_4)_2$ precipitation method was used in plaque analysis of infectious Ad5 DNA (Graham and Van der Eb, 1973).

Enzymes

Xba I was prepared according to the procedure of Zain and Roberts (1977). All other restriction endonucleases were purchased from Bethesda Research Labs, Inc. DNA ligase from bacteriophage T4-infected *E. coli* was purchased from PL Biochemicals. Ligations were performed at 17°C for 16 hr using DNA concentrations greater than 20 μg/ml in the presence of 40 Weiss units per ml of enzyme.

DNAs and Gel Electrophoresis

Viral DNA was prepared from virions as described previously (Jones and Shenk, 1978). Adenovirus DNA-protein complex was prepared by dissolving virions in guanidine hydrochloride (4 M) and centrifuging the complex to equilibrium in CsCl containing guanidine hydrochloride (4 M) (Robinson, Younghusband and Bellett, 1973). DNA fragments

produced by cleavage with restriction endonucleases were separated by electrophoresis in vertical 0.8% agarose slab gels (0.3 × 12 × 24 cm) using Tris–borate buffer [50 mM Tris–OH, 50 mM boric acid, 1 mM EDTA (pH 8.2)].

Selection of Mutants

Mutants lacking Xba I cleavage sites were selected from H5 *sub*304 DNA–protein complex by the procedure of Jones and Shenk (1978). Mutants lacking the Xba I site at 4 map units were also selected by cleavage of H5 mutant 309 DNA–protein complex to completion with this enzyme and plating the digestion product directly onto 293 cells in a DNA plaque assay. The DNA–protein complex used in this selection was prepared from mutant 309 virions which had been serially passaged five times without dilution through 293 cells. The virus was serially passaged on the assumption that this treatment would favor the accumulation of variants containing altered genomes.

Transformation Assays

Primary cultures of rat embryo or rat embryo brain cells were infected at a multiplicity of 10 pfu per cell when the cell monolayers were about 80% confluent. At 24 hr after infection, each plate was subcultured (1:6). At 72 hr after infection, infected cultures were switched from DMEM containing 10% fetal calf serum to MEM containing 0.2 mM $CaCl_2$ and 7% calf serum, and were refed with this low-calcium medium twice a week (Freeman et al., 1967). Foci of transformed cells were evident after 4–5 weeks, and final counts of foci were made at 6–7 weeks.

Acknowledgments

We acknowledge the competent technical assistance of Ms. Bonnie Swerdlow. This work was supported by grants from the American Cancer Society and the USPHS. T.S. is an Established Investigator of the American Heart Association.

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Received April 3, 1979

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